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<p>(54) Title: HUMAN TISSUE INHIBITOR OF METALLOPROTEINASE-4</p>			
<p>(57) Abstract</p> <p>A human tissue inhibitor of metalloproteinases-4 polypeptide and DNA (RNA) encoding such polypeptide and a procedure for producing such polypeptide by recombinant techniques. Also disclosed are methods for utilizing such polypeptide for the treatment of diseases, including arthritis and cancer. Antagonists against such polypeptides and their use as a therapeutic to resorb scar tissue are also disclosed. Diagnostic assays for detecting levels of human TIMP-4 protein and mutations in human TIMP-4 nucleic acid sequence are also disclosed.</p>			

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### HUMAN TISSUE INHIBITOR OF METALLOPROTEINASE-4

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. More particularly, the polypeptides of the present invention are human tissue inhibitor of metalloproteinase-4 polypeptides, hereinafter referred to as "human TIMP-4". The invention also relates to inhibiting the action of such polypeptides.

The extracellular matrix is a complex structure that contains collagen, proteoglycan, glycosaminoglycan, glycoproteins (fibronectin, chondronectin, laminin) and in some tissues, elastin (Hay, E.D., J. Cell Biol., 91:205-223 (1981)).

Matrix metalloproteinases (MMP's) constitute the major group of zinc-binding endopeptidases that degrade extracellular matrix proteins, for example connective tissue, collagen and gelatin, during remodeling of connective tissue during normal physiological and some pathological processes. The unrestrained activity of MMP's may result in extensive tissue damage, and these enzymes have been implicated in a variety of disease processes, including tumor cell invasion, tumor angiogenesis and rheumatoid arthritis (Okada, Y., et

al., J. Biol. Chem., 261:14245-14255 (1986)). The MMP's are secreted from cells as inactive zymogens and their activity in the extracellular environment is regulated by various activators and inhibitors (Matrisian, L.M., Trends Genet., 6:121-125 (1990)).

Regulation of metalloproteinase-mediated proteolysis may occur by naturally occurring inhibitor proteins, such as tissue inhibitor of metalloproteinase (TIMP). The balance between the production and activation of the MMP's, and their inhibition by natural inhibitors such as TIMP, determines, in both physiological and pathological conditions, whether connective tissue is degraded.

MMP's include a number of proteases, exemplified by interstitial (type I) collagenase itself, the stromelysins (also known as proteoglycanases or transins), fibroblast and polymorphonuclear leukocyte gelatinases (also known as collagen-IV-ases), and 'pump-1' (putative metalloproteinases 1, uterine metalloproteinases) [Goldberg et al, J. Biol. Chem. 261:6600 (1986); Whitham et al, Biochem. J. 240:913 (1986); Breathnach et al, Nucleic Acids Res., 15:1139 (1987); Muller et al, Biochem. J., 253:187 (1988); Collier et al, J. Biol. Chem., 263:6579 (1988); Murphy et al, Biochem. J., 258:463 (1989); Quantin et al, Biochem. (N.Y.), 28:5327 (1989); Birkedal-Hansen, J. Oral Pathol., 17:445 (1988)].

In general, the mammalian family of proteases has one or more of the following properties: (a) optimal proteolytic activity around neutral pH; (b) dependence of the enzyme's activity on the presence of zinc, as evident by the loss of activity on treatment with divalent metal ion chelators, such as 1,10 phenanthroline (preferential chelation of zinc), or EDTA (less restricted chelating properties; EDTA and EGTA also contribute to enzyme inactivation via chelation of calcium ions required for enzyme stability); (c) inhibition by TIMPs; (d) absence of significant inhibition by known

inhibitors of other families of neutral, zinc-containing metalloproteases, such as thermolysis, angiotensin-converting enzyme and 'enkephalinases'; and (e) biosynthesis and secretion as latent precursor forms (zymogens), requiring extracellular activation. Activation has been achieved by a number of endoproteases, organomercurials and chaotropic agents.

In general, members of the family of neutral metalloprotease enzymes have distinctive substrate specificities. Thus, collagenase type I is unique in its ability to cleave a specific peptide bond within the natural fibrils of the interstitial collagens (e.g. types I, II and III). The gelatinases are only poorly active on these collagens, but are able to degrade denatured interstitial collagens, as well as the non-fibrillar collagens, e.g. type IV, such as are found in the basement membrane. Pump 1 has been reported to act preferentially on denatured collagens (gelatins), though its profile differs from that of the stromelysins or the collagenases type IV. Both the stromelysins and the gelatinases are also capable of degrading non-collagenous structural proteins, such as the core protein of proteoglycan and elastin. Macromolecules involved in cell-to-substratum and cell-to-cell interactions, such as laminin and fibronectin, are also susceptible to degradation by several of these metalloproteases.

Enzymes of this family are produced by synovial and skin fibroblasts, chondrocytes, peripheral mononuclear cells, keratinocytes and gingival tissue, as well as existing within granule storage vesicles in polymorphonuclear leukocytes (PMNLs).

Current information suggests that there is a family of metalloproteinase inhibitors which comprises TIMP-1 (tissue inhibitor of metalloproteinases-1); TIMP-2; human TIMP-3 which has been cloned, expressed and mapped to human chromosome 22; and chicken tissue inhibitor of

metalloproteinase (ChIMP-5). The polypeptide of the present invention has been putatively identified as a novel human TIMP polypeptide based on amino acid sequence homology.

In accordance with one aspect of the present invention, there is provided a novel mature polypeptide which is human TIMP-4, as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding human TIMP-4, including mRNA's, DNA's, cDNA's, genomic DNA as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptide by recombinant techniques which comprises culturing recombinant prokaryotic and/or eukaryotic host cells, containing a human TIMP-4 nucleic acid sequence under conditions promoting expression of protein and subsequent recovery of said protein.

In accordance with yet a further aspect of the present invention, there is provided a method for treating conditions which are related to insufficient human TIMP-4 activity which comprises administering to a patient in need thereof a pharmaceutical composition containing the human TIMP-4 protein of the invention which is effective to supplement a patient's endogenous human TIMP-4 and thereby alleviate said conditions which include, for example, arthritic diseases such as rheumatoid and osteoarthritis, soft tissue rheumatism, polychondritis and tendonitis; bone resorption diseases, such as osteoporosis, Paget's disease, hyperparathyroidism and cholesteatoma; the enhanced collagen destruction that occurs in association with diabetes; the recessive classes of dystrophic epidermolysis bullosa;

periodontal disease, alveolitis and related consequences of gingival production of collagenase; corneal ulceration; ulceration of the skin and gastro-intestinal tract and abnormal wound healing; post-operative conditions in which collagenase levels are raised; cancer by blocking the destruction of tissue basement membranes leading to cancer metastasis; demyelinating diseases of the central and peripheral nervous systems; asthma; glomerulosclerosis; septic shock and infection; and psoriasis.

In accordance with yet a further aspect of the present invention, there is provided an antibody against such polypeptides.

In accordance with yet another aspect of the present invention, there are provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to human TIMP-4 sequences.

In accordance with yet another aspect of the present invention, there are provided antagonists to such polypeptides which may be employed for therapeutic purposes, for example, for remodeling and repairing tissue and for destruction of scar tissue.

In accordance with another aspect of the present invention, there are provided diagnostic assays for detecting diseases related to mutations in human TIMP-4 sequences and over-expression of the polypeptide.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 shows the cDNA sequence and corresponding deduced amino acid sequence of the full-length human TIMP-4 polypeptide. The standard one-letter abbreviations for amino acids are used. Sequencing was performed using a 373

Automated DNA sequencer (Applied Biosystems, Inc.). Sequencing accuracy is predicted to be greater than 97% accurate.

Figure 2 is an amino acid sequence comparison between the polypeptide of the present invention and other human TIMP polypeptides.

Figure 3 displays the results of a Northern Blot analysis indicating the various human tissues in which human TIMP-4 is expressed.

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figure 1 or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 75946 on November 11, 1994.

A polynucleotide encoding a polypeptide of the present invention may be obtained from an early stage human brain. This contains an open reading frame and coding of protein of 224 amino acid residues of which approximately the first 29 residues represent the leader sequence such that the mature protein comprises 195 amino acid residues. The polynucleotide of this invention was discovered in a cDNA library derived from an early stage human brain. The protein exhibits the highest degree of homology to Human TIMP-2 with 48 % identity and 72 % similarity over a 136 amino acid stretch. Human TIMP-4 has the signature 12 cysteine amino acids, which are conserved in all members of the TIMP family. The 12 cysteine residues are all disulfide-linked in TIMP-1 and TIMP-2. This evidence strongly suggests that the polypeptide of the present invention is a novel member of the TIMP family.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be

the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same, mature polypeptide as the DNA of Figure 1 or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of Figure 1 or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figure 1 or the polypeptide encoded by the

cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in

the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 50% and preferably 70% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNA of Figure 1 or the deposited cDNA.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to a human TIMP-4 polypeptide which has the deduced amino acid sequence of

Figure 1 or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 1 or that encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of Figure 1 or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment

if it is naturally occurring). For example, a naturally occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the human TIMP-4 genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as

vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli lac or trp, the phage lambda  $P_L$  promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Sf9; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant

cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are PKK232-8 and PCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P<sub>R</sub>, P<sub>L</sub> and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a

mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. (Davis, L., Dibner, M., Battey, J., *Basic Methods in Molecular Biology*, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and

a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone"

sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The human TIMP-4 polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography hydroxylapatite chromatography and

lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The present invention is also directed, in part, to human TIMP-4 which has, as a defining characteristic, the ability to inhibit the action of MMP's. The human TIMP-4 polypeptide may be employed as a metalloproteinase inhibitor to prevent tumor invasion and angiogenesis and subsequent metastases. The human TIMP-4 polypeptide may also be employed to treat arthritic diseases, such as rheumatoid arthritis and osteoarthritis, soft tissue rheumatism, polychondritis and tendonitis; and bone resorption diseases, such as osteoporosis, Paget's disease, hyperparathyroidism and cholesteatoma. Human TIMP-4 may also be employed to prevent enhanced collagen destruction that occurs in association with diabetes, the recessive classes of dystrophic epidermolysis bullosa, periodontal disease and related consequences of gingival production of collagenase. Human TIMP-4 may also be employed to inhibit PMNL collagenase release following cellular infiltration to inflamed gingiva, including combatting the greater susceptibility of diabetes patients to periodontal disease.

Human TIMP-4 may also be employed to treat corneal ulceration, for example, that induced by alkali or other

burns, by radiation, by Vitamin E or retinoid deficiency; ulceration of the skin and gastro-intestinal tract, and abnormal wound healing, and post-operative conditions including colonic anastomosis, in which collagenase levels are raised.

MMP's mediate tumor growth *in situ*. Accordingly, human TIMP-4 may be used to block the destruction of cellular basement membranes, which is the mechanism by which cancer cells metastasize. MMP's have been implicated in neovascularization required to support tumor growth and survival, in the tissue remodeling required to accommodate the growing primary and secondary tumors, and in the penetration of tumor cells through the basement membranes of the vascular walls during metastasis.

MMP's are responsible for localized degradation of the follicular wall during ovulation and localized degradation of the uterine wall for blastocyte implantation. Accordingly, human TIMP-4 may be employed as a contraceptive.

Human TIMP-4 may also be employed as a general growth factor to treat restenosis and similar diseases. Human TIMP-4 may be employed particularly as a growth factor for erythroid cell lineages.

Among the other diseases which human TIMP-4 may be employed to treat includes alveolitis, asthma, psoriasis, glomerulosclerosis, and septic shock since MMP's are involved in the tissue invasiveness of some parasites.

Fragments of the full length human TIMP-4 gene may be used as a hybridization probe for a cDNA library to isolate the full length gene and to isolate other genes which have a high sequence similarity to the gene or similar biological activity. Probes of this type can be, for example, between 20 and 2000 bases. Preferably, however, the probes have between 30 and 50 base pairs. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the

complete human TIMP-4 gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the human TIMP-4 gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

This invention is also related to the use of the human TIMP-4 gene as part of a diagnostic assay for detecting diseases or susceptibility to diseases related to the presence of mutated human TIMP-4.

Individuals carrying mutations in the human TIMP-4 gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki et al., *Nature*, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding human TIMP-4 can be used to identify and analyze human TIMP-4 mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled human TIMP-4 RNA or alternatively, radiolabeled human TIMP-4 antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing

agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers et al., *Science*, 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton et al., *PNAS, USA*, 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., *Restriction Fragment Length Polymorphisms (RFLP)*) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

The present invention also relates to a diagnostic assay for detecting altered levels of human TIMP-4 protein in various tissues since an over-expression of the proteins compared to normal control tissue samples may detect the presence of a disease or susceptibility to a disease regulated by human TIMP-4. Assays used to detect levels of human TIMP-4 protein in a sample derived from a host are well-known to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western Blot analysis, ELISA assays and "sandwich" assay. An ELISA assay (Coligan, et al., *Current Protocols in Immunology*, 1(2), Chapter 6, (1991)) initially comprises preparing an antibody specific to the human TIMP-4 antigen, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is

attached a detectable reagent such as radioactivity, fluorescence or, in this example, a horseradish peroxidase enzyme. A sample is removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein like BSA. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any human TIMP-4 proteins attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is now placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to human TIMP-4. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of human TIMP-4 protein present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to human TIMP-4 are attached to a solid support and labeled human TIMP-4 and a sample derived from the host are passed over the solid support and the amount of label detected, for example by liquid scintillation chromatography, can be correlated to a quantity of human TIMP-4 in the sample.

A "sandwich" assay is similar to an ELISA assay. In a "sandwich" assay human TIMP-4 is passed over a solid support and binds to antibody attached to a solid support. A second antibody is then bound to the human TIMP-4. A third antibody which is labeled and specific to the second antibody is then passed over the solid support and binds to the second antibody and an amount can then be quantitated.

This invention also provides a method of screening compounds to identify those which are agonists or antagonists

to be human TIMP-4 polypeptide. An example of such a method comprises obtaining mammalian tissue comprising an extracellular matrix, for example, bovine radiocarpal joints. The articular cartilage is cut into smaller disks and labeled with <sup>35</sup>S-sodium sulfate (10 micro Ci/ml) in DMEM for a sufficient time for the cartilage to incorporate the labeled Sodium sulfate. An MMP, for example, stromelysin, or IL1 or TNF is then added to the cartilage disks under appropriate conditions such that tissue breakdown would normally occur. Human TIMP-4 and the compounds to be screened are then added to the reaction mixture for a sufficient time for the MMP to normally break down the cartilage disks. The supernatant, which is the media outside the cartilage disks, is then collected and radioactivity is counted by a liquid scintillation counter. The percentage of <sup>35</sup>S released into the media is then calculated. This release of <sup>35</sup>S-GAG is representative of the proteoglycan pool in the extracellular matrix of cartilage, and reflects proteoglycan degradation by the MMP. The amount of <sup>35</sup>S-GAG, as determined by liquid scintillation chromatography, is then compared to a control assay done in the absence of the compound to be screened and the ability of the compound to agonize or antagonize the action of human TIMP-4 may then be determined.

Examples of potential human TIMP-4 antagonists, in addition to those identified above, include an antibody, or in some cases, an oligonucleotide, which binds to the polypeptide. Alternatively, a potential antagonist may be a mutated form of human TIMP-4, which recognizes natural substrates, but is inactive, and thereby prevent the action of human TIMP-4.

Potential human TIMP-4 antagonists also include antisense constructs prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to

DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which codes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., *Nucl. Acids Res.*, 6:3073 (1979); Cooney et al, *Science*, 241:456 (1988); and Dervan et al., *Science*, 251: 1360 (1991)), thereby preventing transcription and the production of human TIMP-4. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the human TIMP-4 (antisense - Okano, *J. Neurochem.*, 56:560 (1991); *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of human TIMP-4.

Another potential human TIMP-4 antagonist is a small molecule which binds to and occupies the active site of the human TIMP-4 thereby preventing human TIMP-4 from interacting with MMP's such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules, for example a peptide-bonded molecule.

The human TIMP-4 antagonists may be employed for tissue repair and remodeling, for example, where destruction of scar tissue is desired. In some situations, enhanced connective tissue turnover or remodeling may be desirable, e.g. in resorption of scar tissue; in uterine involution post-partum; in remodeling of fibrotic deposits in the lung, liver or joints. To appropriately control turnover of extra-cellular matrix proteins in these situations would require a balance between the MMP's and human TIMP-4 to appropriately control degradation.

The polypeptides and agonists or antagonists that are also polypeptides may be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

The polypeptides and agonists or antagonists of the present invention may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the pharmaceutical compositions may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intra-articular, intra-tumor, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, the pharmaceutical compositions are administered in an amount of at least about 10  $\mu\text{g}/\text{kg}$  body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day and preferably the dosage is from about 10  $\mu\text{g}/\text{kg}$  to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clones to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 500 or 600 bases; however, clones larger than 2,000 bp have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. FISH requires use of the clones from which the EST was derived, and the longer the better. For example, 2,000 bp is good, 4,000 is better, and more than 4,000 is probably not necessary to get good results a reasonable percentage of the time. For a review of this technique, see Verma et al., *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such

data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native

polyp ptid s. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, *Nature*, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain

sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units to T4 DNA ligase ("ligase") per 0.5  $\mu$ g of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., *Virology*, 52:456-457 (1973).

Example 1

Bacterial Expression and Purification of human TIMP-4

The DNA sequence encoding for human TIMP-4, ATCC #75946, is initially amplified using PCR oligonucleotide primers corresponding to the 5' and sequences of the processed human TIMP-4 protein (minus the signal peptide sequence) and the vector sequences 3' to the TIMP-4 gene. Additional nucleotides corresponding to human TIMP-4 were added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer has the sequence 5' GCCAGAGGATCCTGCAGCTGCAGCCCCGGCAC 3' contains a BamH1 restriction enzyme site followed by 21 nucleotides of human TIMP-4 coding sequence starting from the presumed terminal amino acid of the processed protein codon. The 3' sequence 5' CGGCTTCTAGAACTAGGGCTGAACGATGTCAAC 3' contains an XbaI site and is followed by 18 nucleotides of human TIMP-4. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-9 (Qiagen, Inc. 9259 Eton Avenue, Chatsworth, CA, 91311). pQE-9 encodes antibiotic resistance (Amp'), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-9 was then digested with BamH1 and XbaI. The amplified sequences were ligated into pQE-9 and were inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture was then used to transform E. coli strain m15/pREP4 available from Qiagen by the procedure described in Sambrook, J. et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Laboratory Press, (1989). m15/pREP4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan'). Transformants are identified by their ability to grow on LB plates and

ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation. The cell pellet was solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized human TIMP-4 was purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). Human TIMP-4 (90 % pure) was eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the protein was dialyzed to 10 mmolar sodium phosphate.

#### Example 2

##### Expression of Recombinant human TIMP-4 in COS cells

The expression of human TIMP-4 HA is derived from a vector pcDNA1/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, a SV40 intron and polyadenylation site. A DNA fragment encoding the entire human TIMP-4 precursor and a HA tag fused in frame to its 3' end was cloned into the

polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag correspond to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighten, A. Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37, 767). The fusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows:

The DNA sequence ATCC # 75946, encoding for human TIMP-4 was constructed by PCR using two primers: the 5' primer 5' GCCAGAGGATCCGCCACCATGCCTGGGAGCCCTCGGCC 3' contains a BamHI site followed by 21 nucleotides of human TIMP-4 coding sequence starting from the initiation codon; the 3' sequence 5' CGGCTTCTAGAATCAAGCGTAGTCTGGGACGTCGTATGGGTAGGGCTGAACGATG TCAAC 3' contains complementary sequences to an XbaI site, translation stop codon, HA tag and the last 18 nucleotides of the human TIMP-4 coding sequence (not including the stop codon). Therefore, the PCR product contains a BamHI site, human TIMP-4 coding sequence followed by HA tag fused in frame, a translation termination stop codon next to the HA tag, and an XbaI site. The PCR amplified DNA fragment and the vector, pcDNA1/Amp, were digested with BamHI and XbaI restriction enzyme and ligated. The ligation mixture was transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037) the transformed culture was plated on ampicillin media plates and resistant colonies were selected. Plasmid DNA was isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant human TIMP-4, COS cells were transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring

Laboratory Press, (1989)). The expression of the human TIMP-4 HA protein was detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, (1988)). Cells were labelled for 8 hours with  $^{35}\text{S}$ -cysteine two days post transfection. Culture media were then collected and cells were lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., *Id.* 37:767 (1984)). Both cell lysate and culture media were precipitated with a HA specific monoclonal antibody. Proteins precipitated were analyzed on 15% SDS-PAGE gels.

Example 3

Cloning and expression of TIMP-4 using the baculovirus expression system

The DNA sequence encoding the full length TIMP-4 protein, ATCC # 75946, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence 5' GCCAGAGGGATCC**A**TGCCTGG GAGCCCTCGGCC 3' and contains a BamHI restriction enzyme site (in bold) just behind the first 21 nucleotides of the TIMP-4 gene (the initiation codon for translation "ATG" is underlined).

The 3' primer has the sequence 5' CGGCTTCTAGAACTAGGGCTG AACGATGTCAAC 3' and contains the cleavage site for the restriction endonuclease XbaI and 18 nucleotides complementary to the 3' non-translated sequence of the TIMP-4 gene. The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment was then digested with the endonucleases BamHI and XbaI and then purified again on a 1% agarose gel. This fragment is designated F2.

The vector pA2 (modification of pVL941 vector, discussed below) is used for the expression of the TIMP-4 protein using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases BamHI and XbaI. The polyadenylation site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant viruses the beta-galactosidase gene from *E.coli* is inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for the cell-mediated homologous recombination of co-transfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1 such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., *Virology*, 170:31-39).

The plasmid was digested with the restriction enzymes BamHI and XbaI. The DNA was then isolated from a 1% agarose gel using the commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

Fragment F2 and the plasmid V2 were ligated with T4 DNA ligase. *E.coli* HB101 cells were then transformed and bacteria identified that contained the plasmid (pBacTIMP-4) with the TIMP-4 gene using the enzymes BamHI and XbaI. The sequence of the cloned fragment was confirmed by DNA sequencing.

5  $\mu$ g of the plasmid pBacTIMP-4 was co-transfected with 1.0  $\mu$ g of a commercially available linearized baculovirus ("BaculoGold" baculovirus DNA", Pharmingen, San Diego, CA.)

using the lipofection method (Felgner et al. Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987)).

1 $\mu$ g of BaculoGold™ virus DNA and 5  $\mu$ g of the plasmid pBacTIMP-4 were mixed in a sterile well of a microtiter plate containing 50  $\mu$ l of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10  $\mu$ l Lipofectin plus 90  $\mu$ l Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added drop-wise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate was rocked back and forth to mix the newly added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours the transfection solution was removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant was collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution, the viruses were added to the cells and blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses was then resuspended in an Eppendorf tube containing 200  $\mu$ l of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculovirus was used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the recombinant baculovirus V-TIMP-4 at a multiplicity of infection (MOI) of 2. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5  $\mu$ Ci of  $^{35}$ S-methionine and 5  $\mu$ Ci  $^{35}$ S cysteine (Amersham) were added. The cells were further incubated for 16 hours before they were harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

Example 4

Expression pattern of Human TIMP-4 in human tissues

20  $\mu$ g of total RNA from each of the above tissues was denatured and run on a 1.2% formaldehyde agarose gel and capillary blotted onto a nylon filter overnight. RNA was immobilized on the filter by UV cross-linking. A random primer probe was prepared from the EcoRI-Xhol insert of the partial TIMP-4 nucleic acid sequence and used to probe the blot by overnight hybridization in Church buffer with 100  $\mu$ g/ml denatured herring sperm DNA as a blocking agent. Washing was performed sequentially with 2 x SSC/0.1% SDA and 0.2xSSC/0.1% SDS at 65 degrees Celsius. Size markers were the BRL RNA ladder and 18S and 28S ribosomal RNA bands.

Figure 3.

## SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: GREENE, ET AL.

(ii) TITLE OF INVENTION: Human TIMP-4

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN,  
CECCHI, STEWART & OLSTEIN

(B) STREET: 6 BECKER FARM ROAD

(C) CITY: ROSELAND

(D) STATE: NEW JERSEY

(E) COUNTRY: USA

(F) ZIP: 07068

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 INCH DISKETTE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: MS-DOS

(D) SOFTWARE: WORD PERFECT 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: Submitted herewith

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA

(A) APPLICATION NUMBER:

(B) FILING DATE:

## (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: FERRARO, GREGORY D.
- (B) REGISTRATION NUMBER: 36,134
- (C) REFERENCE/DOCKET NUMBER: 325800-278

## (ix) TELECOMMUNICATION INFORMATION:

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- (B) TELEFAX: 201-994-1744

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 675 BASE PAIRS
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGCCTGGGA	GCCCTCGGCC	CGCGCCAAGC	TGGGTGCTGT	TGCTGCGGCT	GCTGGCGTTG	60
CTGCGGCC	CGGGGCTGGG	TGAGGCATGC	AGCTGCGCCC	CGGCGCACCC	TCAGCAGCAG	120
ATCTGCCACT	CGGCACCTGT	GATTGGGCC	AAAATCTCCA	GTGAGAAGGT	AGTTCCGGCC	180
AGTGCAGACC	CTGCTGACAC	TGAAAAAAATG	CTCCGGTATG	AAATCAAACA	GATAAAGATG	240
TTCAAAGGGT	TTGAGAAAGT	CAAGGATGTT	CAGTATATCT	ATACGCCCTT	TGACTCTTCC	300
CTCTGTGGTG	TGAAACTAGA	AGCCAACAGC	CAGAAGCAGT	ATCTCTTGAC	TGGTCAGGTC	360
CTCAGTGATG	GAAAAGTCTT	CATCCATCTG	TGCAAGTACA	TCGAGCCCTG	GGAGGACCTG	420
TCCCTGGTGC	AGAGGGAAAG	TCTGAATCAT	CACTACCATC	TGAACGTGTT	CTGCCAAATC	480
ACCAACCTGCT	ACACAGTACC	CTGTACCATC	TCGGCCCCTA	ACGAGTCCT	CTGGACAGAC	540
TGGCTGTTGG	AACGAAAGCT	CTATGGTTAC	CAGGCTCAGC	ATTATGTCTG	TATGAAGCAT	600
CTTGACTTCA	CCTGCAGCTG	GTACCGGGGC	CACCTGCCTC	TCAGGAAGGA	GTTTGTGAC	660
ATXGTTCA	CCTAG					675

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 224 AMINO ACIDS

(B) TYPE: AMINO ACID

**(C) STRANDEDNESS.**

(B) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3.

Trp Leu Leu Glu Arg Lys Leu Tyr Gly Tyr Gln Ala Gln His Tyr  
155 160 165  
Val Cys Met Lys His Val Asp Gly Thr Cys Ser Trp Tyr Arg Gly  
170 175 180  
His Leu Pro Leu Arg Lys Glu Phe Val Asp Ile Val Gln Pro  
185 190 195

WHAT IS CLAIMED IS:

1. An isolated polynucleotide selected from the groups consisting of
  - (a) a polynucleotide encoding a polypeptide having the deduced amino acid sequence of Figure 1 or a fragment, analog or derivative of said polypeptide;
  - (b) a polynucleotide encoding a polypeptide having the amino acid sequence encoded by the cDNA contained in ATCC Deposit No. 75946 or a fragment, analog or derivative of said polypeptide.
2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.
3. The polynucleotide of Claim 1 wherein the polynucleotide is RNA.
4. The polynucleotide of Claim 1 wherein the polynucleotide is genomic DNA.
5. The polynucleotide of Claim 2 wherein said polynucleotide encodes a polypeptide having the deduced amino acid sequence of Figure 1.
6. The polynucleotide of Claim 2 wherein said polynucleotide encodes a polypeptide encoded by the cDNA of ATCC Deposit No. 75946.
7. The polynucleotide of Claim 1 having the coding sequence as shown in Figure 1.
8. The polynucleotide of Claim 2 having the coding sequence deposited as ATCC Deposit No. 75946.
9. A vector containing the DNA of Claim 2.
10. A host cell genetically engineered with the vector of Claim 9.
11. A process for producing a polypeptide comprising: expressing from the host cell of Claim 10 the polypeptide encoded by said DNA.
12. A process for producing cells capable of expressing a polypeptide comprising genetically engineering cells with the vector of Claim 9.

13. An isolated DNA hybridizable to the DNA of Claim 2 and encoding a polypeptide having human TIMP-4 activity.
14. A polypeptide selected from the group consisting of (i) a polypeptide having the deduced amino acid sequence of Figure 1 and fragments, analogs and derivatives thereof and (ii) a polypeptide encoded by the cDNA of ATCC Deposit No. 75946 and fragments, analogs and derivatives of said polypeptide.
15. The polypeptide of Claim 14 wherein the polypeptide has the deduced amino acid sequence of Figure 1.
16. An antibody against the polypeptide of claim 14.
17. An antagonist against the polypeptide of claim 14.
18. A method for the treatment of a patient having need of human TIMP-4 comprising: administering to the patient a therapeutically effective amount of the polypeptide of claim 14.
19. A method for the treatment of a patient having need to inhibit human TIMP-4 comprising: administering to the patient a therapeutically effective amount of the antagonist of Claim 17.
20. A pharmaceutical composition comprising the polypeptide of Claim 14 and a pharmaceutically acceptable carrier.
21. The method of Claim 18 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.
22. A process for identifying compounds active as agonists or antagonists to human TIMP-4 comprising: combining an MMP, human TIMP-4, a compound to be screened and a reaction mixture containing a substrate capable of degradation by the MMP, wherein said substrate is labeled; and

determining the ability of the compound to enhance or block the degradation of the substrate by said MMP by measuring the label released from the substrate.

23. A process for diagnosing a disease or a susceptibility to a disease related to a mutation in human TIMP-4 nucleic acid sequence comprising:

isolating a nucleic acid sequence encoding human TIMP-4 from a sample derived from a host; and

determining a mutation in the human TIMP-4 nucleic acid sequence.

24. A diagnostic process comprising:

analyzing for the presence of the polypeptide of claim 14 in a sample derived from a host.

1 / 3

1 ATGCCTGGGAGCCCTGCGGGGGGAGACAAAGCTGGGTGGCTGTT  
 M P G S P R P A P S W V L L  
 42 GCTGGGGCTGGCTGGCGTGGCTGGGGGGGGGGCTGGGTGG  
 L R L L A L L R P P G L G  
 83 AGGCATGCAGCTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG  
 E A C S C A P A H P Q Q H I  
 124 TGCCACTGGCACTTGATGGGACAAATCTCCAGGAGA  
 C H S A L V I R A K I S S E  
 165 GAAGGTAGTTCGGGAGTGGCAGACCCCTGGTGGACACTGAAA  
 K V V P A S A D P A D T E  
 206 AAATGCTCCGGTATGAAATCAAACAGATAAAGATGGTCAAA  
 K M L R Y E I K Q I K M F K  
 247 GGGTTTGAGAAAGTCAAGGAATGGTCAAGTATATCTATAAGGC  
 G F E K V K D V Q Y I Y T P  
 288 TTTGACTCTCCCTCTGGGTGTGAAACTAGAAGCCAACA  
 F D S S L C G V K L E A N  
 329 GCGAGAAGCAGTATCTCTGACTGGTCAGGTCCTCAGTGGAT  
 S Q K Q Y L L T G Q V L S D  
 370 GGAAAAGTCTCATCCATCTGGCAACTACATCGAGGCGCTG  
 G K V F I H L C N Y I E P W  
 411 GGAGGACCTGGCTGGTGGCAGAGGGAAAGTCAGAATCATC  
 E D L S L V Q R E S L N H  
 452 ACTACCATCTGAACCTGGCTGGCAAAATCACCCACCTGCTAC  
 H Y H L N C G C Q I T T C Y  
 493 ACAGTACCCCTGTACCATCTGGGGGGCTAACGAGTGGCTCTG  
 T V P C T I S A P N E C L W  
 534 GACAGACTGGCTGGAAACGAAAGCTCTATGGTACCAAGG  
 T D W L L E R K L Y G Y Q  
 575 CTCAGCATTATGCTGTTATGAACCATGGTGGCACCTGCG  
 A Q H Y V C M K H V D G T C  
 616 AGCTGGTACCGGGGGCCACCTGGCTCTCAGGAAGGAGTTGT  
 S W Y R G H L P L R K E F V  
 657 TGACATCGTCAAGCCCTAG

FIG. I

D I V Q P .

TIMP.msf (Human_TIMP2)	MGAARTLRL	ALGLILLATL	LRP..A..DA	CSCSPVHPQQ	AFCNADVVIR	50
TIMP.msf (Human_TIMP4)	MPGSPPAPs	WVLLIRLLAL	LRPPGLG.EA	CSCAPAHPPQQ	HICHSAVIR	
TIMP.msf (Human_TIMP3)	..MTP	WLGILVLLGS	WSLGDWGAEA	CTCSPSHPQQ	AFCNSDIVIR	
TIMP.msf (Human_TIMP1)	MAPFEPLASG	ILLLWLIAP	SR..A..A	CTCVPHPQT	AFCNSDLVIR	
Consensus	-----	-----	-----	-----	-----	
TIMP.msf (Human_TIMP2)	AKAVSEKEV	SGNDIYGNPI	KRIQYEIKQI	KMFKGPE..	..KDIIFIYT	100
TIMP.msf (Human_TIMP4)	AKISSEKVV	ASADP.ADTE	KMLRYEIKQI	KMFKGFEKV.	..KDVQYIYT	
TIMP.msf (Human_TIMP3)	AKVVGKKLVK	EG..PF	GTLVYTIKOM	KMYRGFTKM.	..PHVQYIHT	
TIMP.msf (Human_TIMP1)	AKFVGTPEVN	....QTT	LYQRYEIKMT	KMYKGFQALG	DAADIRFVYT	
Consensus	AK-----V-	-----	-----	-----	-----	
TIMP.msf (Human_TIMP2)	APSSAVCGVS	LDVGGKKEYL	LAGKAEGDGK	MHITLCDFIV	PWDTLSTTQR	150
TIMP.msf (Human_TIMP4)	PFDSSLICGVK	LEANSQKQYL	LTGQVLSDGK	VFIHLCNYIE	PWEDLSLVQR	
TIMP.msf (Human_TIMP3)	EASESLCGLK	LEVN.KYQYL	LTGRVY.DGK	MYTGLCNFVE	RWDQTLTSQR	
TIMP.msf (Human_TIMP1)	PAMESVCGYF	HRSHNRSEEF	LIAGKLQDGL	LHITTCSFVA	PWNLSLAQR	
Consensus	-----CG--	-----	-----DG--	-----C--	-----W--L--Q--	
TIMP.msf (Human_TIMP2)	KSLNHRYQMG	C.ECKITRCP	MIPCYISSPD	ECLWMDWVTE	KNINGHQAKF	200
TIMP.msf (Human_TIMP4)	ESLNHHYHHLN	C.GCQITTCY	TVPCTISAPN	ECLWTDWILLE	RKLYGYQAQH	
TIMP.msf (Human_TIMP3)	KGLNRYHHLG	C.NCKIKSCY	YLPCCFTTSKN	ECLWTDMLSN	FGYPGYQSKH	
TIMP.msf (Human_TIMP1)	RGFTKTYTVG	CEECTVFPC	SIPCKLQSGT	HCLWTDQLLQ	GSEKGFQSRH	
Consensus	-----Y--C--	-----C--	-----PC--	-----CLW-D--	-----G-Q--	
TIMP.msf (Human_TIMP2)	FACIKRSDGS	CAW..YRGAA	PPKQEFLDIE	DP	232	
TIMP.msf (Human_TIMP4)	YVCMKHVDG	CSW..YRGHL	PLRKEFVDIV	QP		
TIMP.msf (Human_TIMP3)	YACIRQKGGY	CSW..YRGWA	PPDKSIIINAT	DP		
TIMP.msf (Human_TIMP1)	LACLPRPGL	CTWQSLRSQI	A.....	..		
Consensus	-----C-----	-----C-W--R--	-----	-----		

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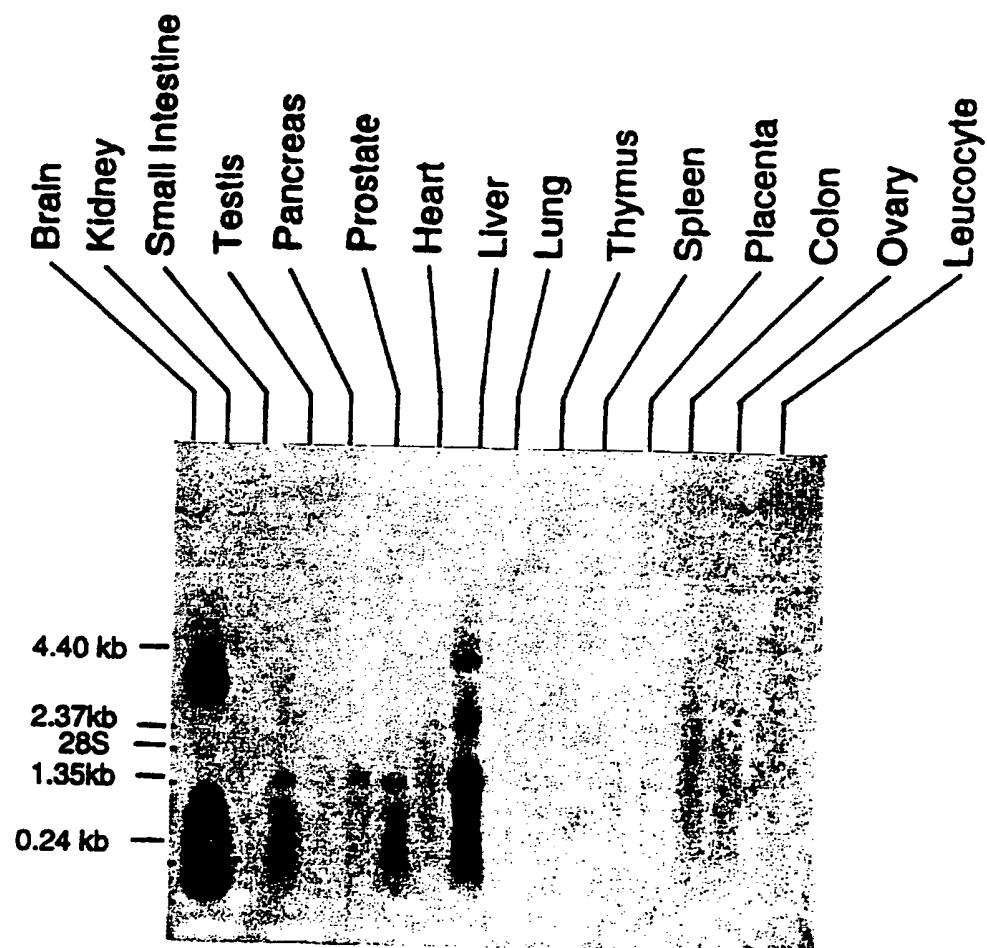


FIG. 3

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/14498

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :435/23, 69.2, 240.2, 252.3, 320.1; 536/23.5; 530/350; 514/12, 44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/23, 69.2, 240.2, 252.3, 320.1; 536/23.5; 530/350; 514/12, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, BIOSIS, MEDLINE, LIFESCI, EMBASE, BIOTECHDS, CANCERLIT, WPI  
search terms: timp, gene# or sequence#, timp-4,

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CANCER RESEARCH, Volume 54, issued 15 April 1994, J.A. Uria et al., "Structure and Expression in Breast Tumors of Human TIMP-3, a New Member of the Metalloproteinase Inhibitor Family", pages 2091-2094, see entire document.	1-15, 18, 20-23
Y	WO, A, 90/11287 (STETLER-STEVENSON ET AL.) 04 October 1990, entire document.	1-15, 18, 20-23
Y	BIOTECHNOLOGY, Volume 5, issued June 1987, M. Kaczorek et al., "Molecular Cloning and Synthesis of Biologically Active Human Tissue Inhibitor of Metalloproteinases in Yeast", pages 595-598, see entire document.	1-15, 18, 20-23

<input type="checkbox"/>	Further documents are listed in the continuation of Box C.	<input type="checkbox"/>	See patent family annex.
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* Special categories of cited documents:	
*A*	document defining the general state of the art which is not considered to be of particular relevance
*E*	earlier document published on or after the international filing date
*L*	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
*O*	document referring to an oral disclosure, use, exhibition or other means
*P*	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"A"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 MAY 1995	08 MAY 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>Deborah Freese</i> REBECCA PROUTY Telephone No. (703) 308-0196

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US94/14498

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-15, 18, 20-23

**Remark on Protest**

The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US94/14498

**A. CLASSIFICATION OF SUBJECT MATTER:**  
IPC (6):

C12N 1/21, 5/10, 15/09, 15/15, 15/63; C07K 14/81; A61K 38/57, 48/00; C12Q 1/37

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-15, 18, and 20-23, drawn to human TIMP-4, DNA encoding TIMP-4 and uses thereof.

Group II, claims 16 and 24, drawn to human TIMP-4 antibody and uses thereof.

Group III, claims 17 and 19, drawn to an antagonist to human TIMP-4 and uses thereof.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The compounds of Groups I-III are chemically unrelated comprising unrelated chemical structures.

